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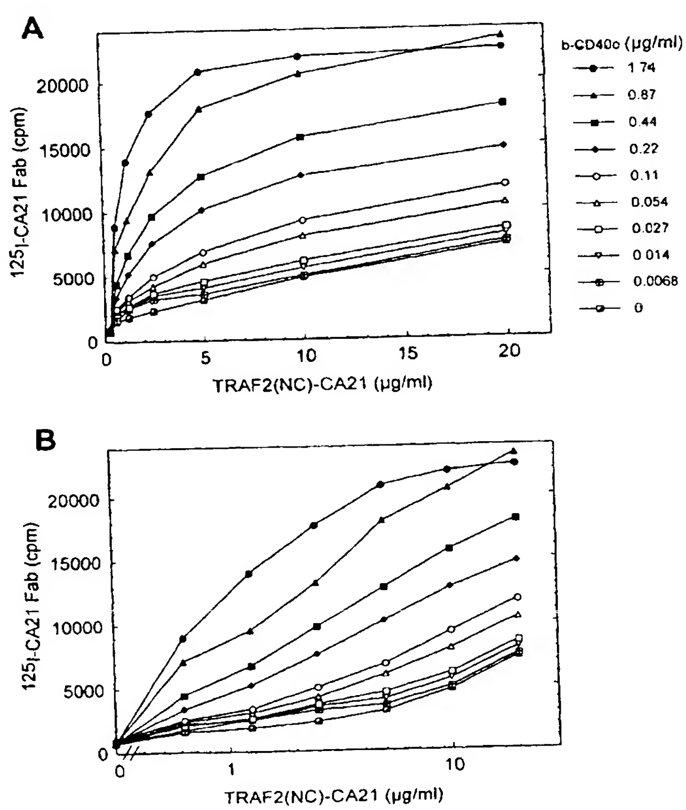
(57) Abstract

Disclosed is a high throughput compatible assay that is useful for the identification of specific antagonists of TRAF–receptor interactions. The modular flexibility of the assay makes it possible to introduce simple modifications in order to measure the interaction of any TNF receptor cytoplasmic domain (or TRAF-binding protein) with any of the six TRAF proteins, TRAF1, TRAF2, TRAF3, TRAF4, TRAF5 and TRAF6.



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ASSAY FOR RECEPTOR–TRAF INTERACTIONS

This application claims benefit to US Application No. 09/181,958 filed October 29, 1998.

Background of the Invention

1. Field of the Invention

The invention relates to screening assays for compounds effecting TRAF-receptor interactions and is useful for the identification of agonists and antagonists of TRAF-receptor interactions.

2. Description of Related Art

Tumor necrosis factor (TNF) receptor superfamily members regulate cellular proliferation, differentiation, and apoptosis in inflammatory and immune responses. Signaling through TNF receptor superfamily members is initiated by oligomerization of the receptors with trimeric ligands bringing intracellular domains in close proximity. Signal transduction through many of these receptors is mediated in part by a recently identified family of proteins termed TNF receptor-associated factors (TRAFs). Six TRAF family members have been identified. Cao, Z., et al (1996) *Nature 383*, 443-446; Cheng, G. et al 995 *Science 267*, 1494-1498; Rothe, M., et al (1994) *Cell 78*, 681-692; Sato, T., et al (1995) *FEBS Letters 358*, 113-118; Hu, H. M., et al (1994) *J. Biol. Chem. 269*, 30069-30072; Mosialos, G., et al (1995) *Cell 80*, 389-399; Regnier, C. H., et al (1995) *J. Biol. Chem. 270*, 25715-25721; Nakano, H., et al (1996) *J. Biol. Chem. 271*, 14661-14664. Subsets of TRAF proteins have been shown to interact with the TNF receptor family members TNFR2, CD40, CD30, LTßR, ATAR, OX-40, and 4-1BB. Cheng et al (1995); Rothe et al (1994); Sato et al (1995); Hu et al (1994); Nakano et al (1996); Ishida, T. K., et al (1996) *Proc. Natl. Acad. Sci. U. S. A. 93*, 9437-9442; Ishida, T., et al (1996) *J. Biol. Chem. 271*, 28745-28748; Boucher, L. M., et al (1997) *Biochem. Biophys. Res. Commun. 233*, 592-600; Lee, S. Y., et al (1996) *Proc. Natl. Acad. Sci. U. S. A. 93*, 9699-9703; Gedrich, R. W., et al (1996) *J. Biol. Chem. 271*, 12852-12858;

Marsters, S. A., et al (1997) *J. Biol. Chem.* 272, 14029-14032; Aizawa, S. et al (1997) *J. Biol. Chem.* 272, 2042-2045; Arch, R. H. et al (1998) *Molec. Cell Biol.* 18, 558-565; Devergne, O., et al (1996) *Molec. Cell Biol.* 16, 7098-7108; VanArsdale, T. L., et al (1997) *Proc. Natl. Acad. Sci. U.S.A.* 94, 2460-2465. The conserved C-terminal region of TRAFs (TRAF(NC), also delineated the TRAF domain) binds to receptor cytoplasmic domains. Cheng et al (1995); Takeuchi, M., et al (1996) *J. Biol. Chem.* 271, 19935-19942; Rothe, M., et al (1995) *Science* 269, 1424-1427. The initial event in signaling is thought to be mediated by a transient recruitment of TRAF proteins following receptor cross-linking. Kuhne, M. R., et al (1997) *J. Exp. Med.* 186, 337-342. The interaction of TRAF proteins with cross-linked receptor cytoplasmic domains is therefore a critical step in TNF receptor family member signaling and determines the specificity of the resulting cellular response.

Thus, it is apparent that there is a clear need for a quantitative binding assay for TRAF-receptor interactions and which has the modular flexibility to make possible the introduction of simple modifications in order to measure the interaction of any TNF receptor cytoplasmic domain (or TRAF-binding protein) with any of the six TRAF proteins. Such an assay would be useful for identification of specific agonists or antagonists of these interactions.

Summary of the Invention

All of the above factors provide a strong incentive for the development of an efficient, accurate and reproducible assay capable of quantifying TRAF and TRAF interacting receptor binding. The assays of the present invention are useful in pharmacological studies of these interactions and provide an efficient alternative to the use of receptor – TRAF co-precipitation assays or yeast two hybrid interaction assays. It is therefore an object of the present invention to provide a quantitative assay for measuring the ability of a substance to effect binding of a TRAF protein to a TRAF interacting receptor, the assay performed by providing the receptor having a cytosolic binding domain or fragment thereof, wherein the receptor is bound to a multi-well plate, the multi-well plate being capable of allowing formation of a multimeric receptor and

being capable of allowing detection of a signal; providing a TRAF protein or fragment thereof possessing a terminal tag, the tag having one or more amino acids and being capable of binding a signal-generating antibody or fragment thereof: providing the substance: combining the receptor bound to the well, the TRAF protein and the substance: and detecting the signal-generating antibody.

In certain specific embodiments, there are provided quantitative assays according to the invention with specific TRAF proteins and their respective receptors, and a multi-well plate having a scintillant and a signal-generating antibody which has a radioactive isotope.

Description of the Drawings

Figure 1. Two-way titration of biotin-CD40c and TRAF2(NC)-CA21. Streptavidin flashplates were coated with serial dilutions of biotin-CD40c, starting at 1.74 μg/ml. Purified TRAF2(NC)-CA21 was serially diluted starting at 20 μg/ml. Detection was with ^{125}I-CA21 Fab (0.08 μCi/well). Background was not subtracted and is listed as biotin-CD40c at 0 μg/ml. Biotin-CD40c concentrations are indicated. A) linear plot; B) log plot of TRAF2(NC)-CA21 concentration for the same experiment in A).

Figure 2. Peptide antagonists of CD40c-TRAF2 interaction. Streptavidin flashplates were coated with 0.44 μg/ml biotin-CD40c. Stock solutions of peptides were prepared at 1 mg/ml in assay buffer and the pH verified. All peptides were assayed by preincubating with TRAF2(NC)-CA21 at a final peptide concentration of 0.5 mg/ml. Peptides were serially diluted 1.5-fold prior to addition of the TRAF2(NC)-CA21 (5 μg/ml final). Results are means of duplicate points except for the no inhibitor wells (none, closed circles) that are means ± standard deviation of 11 replicates. A) Peptides AcPVQET-NH2 (closed squares), AcNTAAPVQET-NH2 (closed triangles), AcNTAAPVQETLH-NH2 (open triangles), CD30.B (AcDVMLSVEEEGKE-NH2, open circles); B) Peptides AcIQET-NH2 (closed squares), AcPIQET-NH2 (closed triangles), AcQEPQEINF-NH2 (open triangles), CD40c (open circles). The maximum signal in B) is less due to a difference in the decay of the ^{125}I-Fab.

Detailed Description of the Invention

The assay according to the invention, is a high throughput compatible assay that is useful for the identification of specific antagonists of TRAF-receptor interactions. The modular flexibility of the assay makes it possible to introduce simple modifications in order to measure the interaction of any TNF receptor cytoplasmic domain (or TRAF-binding protein) with any of the six TRAF proteins, TRAF1, TRAF2, TRAF3, TRAF4, TRAF5 and TRAF6.

The multi-well plate according to the invention is any multi-well plate capable of allowing detection of a signal. For example, in a preferred embodiment, a flashplate design was chosen for the assay. In a flashplate design, a scintillant-embedded 96-well plate precoated with a substance capable of multimerizing the receptor such as streptavidin.

The assay according to the invention can use a TRAF interacting receptor such as ATAR, LT-ßR, TNFR2, CD40, CD30, OX-40 and 4-1BB, preferably CD40. The 62 amino acid CD40 cytoplasmic domain (CD40c) was expressed in *E. coli* and purified as described in Example 2 below. Analytical ultracentrifugation studies showed that the CD40c protein was a monomer. CD40c had a weak ability to compete for GST-CD40-TRAF interaction. Pullen, S. S., et al (1998) *Biochem.*, 37 11836-11845. When coated on standard 96-well plates and used to bind TRAF molecules the CD40c gave a weaker signal than dimeric GST-CD40. Therefore, to design the CD40c-TRAF interaction assay, CD40c was conjugated with biotin. Streptavidin-coated wells were used to multimerize CD40c, each streptavidin tetramer being capable of binding a maximum of four biotin-CD40c molecules. This approach was predicted to mimic the results of receptor cross-linking by trimeric ligands.

To establish a uniform signal for TRAF binding that would not be amplified during detection by artificial (antibody-induced) cross-linking, defined and purified protein components are preferred . Because of the difficulty in purifying recombinant full-length TRAF, the present invention utilizes TRAF(NC) domains, preferably of TRAF2 and TRAF3, possessing a C-terminal tag with a nine amino acid epitope that is

recognized by the CA21 monoclonal antibody. Kahn, J., et al (1994) *J. Cell Biol. 125*, 461-470. The TRAF2(NC)-CA21 and TRAF3(NC)-CA21 proteins as shown in sequence nos. 1 and 2, respectively, were expressed in insect cells and purified by ion exchange chromatography and hydroxyapatite chromatography as described herein. Chemical cross-linking, analytical ultracentrifugation and laser light scattering methods demonstrated that the TRAF(NC) domains of TRAF2 and TRAF3 formed homogeneous noncovalent trimers.

Detection of the bound TRAF(NC) proteins is performed with a signal-generating antibody. A signal-generating antibody is any antibody or fragment thereof possessing specificity for the tag as described herein. The signal-generating antibody is also capable of generating a signal by means known in the art, for example, by possessing a fluorophore or radiolabel. In a preferred embodiment, the signal-generating antibody is a Fab fragment of the CA21 monoclonal antibody. The CA21 Fab can be radiolabeled with, for example, radioactive iodine as described herein, and the specific activity adjusted continuously for radioactive decay according to the half life of the radiolabel. Radioactivity bound to the scintillant-embedded wells indicated TRAF binding and could be detected with minimal background by scintillation counting the plates without removal of the radioactive solution.

To establish and optimize the assay parameters for the detection of signaling inhibitors it was desired to obtain a suboptimal signal while retaining a good signal/noise ratio. Additionally, it was desired to minimize the quantity of TRAF interacting receptor and obtain a good dynamic range of signal. In this design, inhibitors of CD40-TRAF interaction as well as enhancers/stabilizers of the interaction could be readily detected. Since CD40-TRAF interaction is thought to be transient (Kuhne et al (1997)), both inhibitors and enhancers of the interaction would be expected to antagonize cellular signaling.

The assay parameters were established with a two-way titration of biotin-CD40c and TRAF2(NC)-CA21, as shown in Figure 1. The assay showed saturation with increasing concentrations of TRAF2(NC)-CA21, and gave a good dose response for biotin-CD40c. Maximal signal was obtained with approximately 2 μg/ml biotin-CD40c, and the signal did not increase further up to 20 μg/ml. Significant signal was detectable

as low as 0.054 μg/ml biotin-CD40c (Figure 1). Standard conditions for the assay were selected to be 0.44 μg/ml CD40c and 5 μg/ml TRAF2(NC)-CA21. These conditions provided a signal/noise of eight to ten and a good dynamic range for the detection of inhibitors or enhancers. DMSO at concentrations up to 10% (v/v) did not inhibit the assay. As noted by the manufacturer of the plates, low concentrations of detergents were strong inhibitors of the assay because they removed the streptavidin from the wells. To demonstrate the use and specificity of the assay several N-terminally acetylated and C-terminally amidated peptides derived from the cytoplasmic domains of CD40 and CD30 were assayed for the ability to inhibit CD40c-TRAF2 interaction. The PVQET sequence in the CD40 cytoplasmic domain is essential for signaling through CD40 and is thought to be a core TRAF2 binding sequence. Devergne, O., et al (1996) *Mol. Cell. Biol. 16*, 7098-7108; Innui, W., et al (1990) *Eur. J. Immunol. 20*, 1747-1753. Therefore several peptides around the PVQET sequence were tested for the ability to compete the CD40c-TRAF2 interaction (Figure 2A). Three different peptides containing the PVQET sequence were found to inhibit CD40c-TRAF2 binding. The longest peptide, an 11-mer, was the least potent, and the shortest peptide, PVQET, was the most potent, with an IC_{50} of approximately 90 μM. The activity of PVQET was comparable to a 12 amino acid residue TRAF2-binding peptide derived from the CD30 cytoplasmic domain (Boucher et al (1997)). Figure 2A. The nonbiotinylated CD40c polypeptide had an IC_{50} of approximately 50 μM. A CD40-derived peptide non-overlapping with the PVQET peptide that has been demonstrated to bind TRAF6 (26) did not inhibit CD40c-TRAF2 interaction (Figure 2B). Alteration of the PVQET sequence to PIQET resulted in a slightly increased inhibitory activity (IC_{50} ~70 μM), as predicted by amino acid replacement analyses on the TRAF2 binding peptide derived from CD40. Additionally, removal of the ^{250}Pro residue to produce a four amino acid residue peptide, IQET, resulted in a approximately two-fold decrease of inhibitory activity (IC_{50} ~140 μM) (Figure 2B).

Similar assay results have also been obtained with the same peptide competitors using 5 μg/ml TRAF3(NC)-CA21 instead of TRAF2(NC)-CA21. Thus, it would be expected that either TRAF1(NC)-CA21 or TRAF6(NC)-CA21 could be also substituted for TRAF2 in binding to biotin-CD40c. Pullen et al (1998). Alternatively, biotin-

conjugated cytoplasmic domains of other TRAF-interacting receptors such as ATAR/HVEM or LT- R could be substituted for biotin-CD40c. The alternative of using a ^{3}H-Fab fragment is also a possibility that would decrease the need for frequent radio-iodinations. These variations on the assay show its adaptability and utility as a specificity assay. For example, in a set of assays measuring CD40c-TRAF2, CD40c-TRAF3, ATARc-TRAF2, or ATARc-TRAF3 interaction, it could readily be determined whether an inhibitor targeted CD40c, ATARc, TRAF2, or TRAF3, or was nonspecific.

As will be appreciated by those skilled in the art, the assay can be used to derive peptide-based as well as non-peptide small molecule antagonists of TRAF-mediated signaling. Targets would include receptor cytoplasmic domains or individual TRAF proteins.

In order that this invention be more fully understood, the following examples are set forth. These examples are for the purpose of illustrating preferred embodiments of this invention, and are not to be construed as limiting the scope of the invention in any way.

All references cited in this application are fully incorporated by reference.

Example 1

Plasmids and viruses

The human CD40 cytoplasmic domain, amino acids 216-277, was PCR amplified using oligonucleotides 5′-CCGGGCCATGGCCAAAAAGGTGGCCAAGAAGCCAACC-3′ and 5′-CCCGGGAATTCTCATCACTGTCTCTCCTGCACTGAGATGCG-3′and ligated into pCR2.1 (InVitrogen) to generate pCD40c. The *Nco*I to *EcoR*I fragment was ligated into pET-23d to generate pCD40c/pET23d.

Full-length human TRAF2 and TRAF3 were PCR amplified from a PHA-stimulated human peripheral blood leukocyte cDNA library (Clontech) using oligonucleotides 5′-AAAAGGAAAAGCGGCCGCTTATTAGAGCCCTGTCAGGTCCA-3′ and 5′-TTGGTTGGATCCTATAAATATGGCTGCAGCTAGCGTGA-3′ for TRAF2 and

oligonucleotides 5´-TTGGTTGGATCCTATAAATATGGAGTCGAGTAAAAAGATGGACTC-3´ and 5´-GCGGCCGCTCATCAGGGATCGGGCAGATCCGA-3´ for TRAF3, and ligated into pGem-T (Promega) to make pTRAF2/GemT and pTRAF3/GemT, respectively. The TRAF2(NC) domain (amino acids 272-501) and TRAF3(NC) domain (amino acids 354-568) were PCR amplified from pTRAF2/GemT and pTRAF3/GemT, respectively, using oligonucleotides 5´-CCATGGCCTGCGAGAGCCTGGAGAAGAAGACGGCCACTTTTGA-3´ and 5´-AAAAGGAAAAGCGGCCGCTTATTAGAGCCCTGTCAGGTCCA-3´ for TRAF2(NC) and oligonucleotides 5´-CCATGGTGGAGTCCCTCCAGAACCGCGTGACCGAGCT-3´ and 5´-GCGGCCGCTCATCAGGGATCGGGCAGATCCGA-3´ for TRAF3(NC) respectively, and ligated into pGem-T to create pTRAF2(NC)/GemT and pTRAF3(NC)/GemT, respectively. An *Nco*I linker (CCCATGGG) (New England Biolabs) was ligated into the transplacement vector pVL1393 (InVitrogen) after digestion with *Sma*I to create pVL1393/NcoI^{+}. The *Nco*I to *Not*I TRAF(NC) domain-containing fragments from pTRAF2(NC)/GemT and pTRAF3(NC)/GemT were ligated into pVL1393/NcoI^{+} to create pTRAF2(NC)/1393 and pTRAF3(NC)/1393, respectively. The TRAF(NC) domain constructs of TRAF2 and TRAF3 were C-terminally tagged with the nine amino acid epitope (SKRSMNDPY) recognized by the CA21 monoclonal antibody (Kahn et al (1994)) by PCR methods to generate TRAF2(NC)-CA21 and TRAF3(NC)-CA21 in pVL1393. Recombinant baculovirus stocks were generated by standard methods from the transplacement vectors described above. O'Reilly, D. R., Miller, L. K., and Luckow, V. A. (1992) *Baculovirus expression vectors: a laboratory manual.*, W. H. Freeman & Co., Salt Lake City, UT.

Example 2

Protein expression and purification

Spodoptera frugiperda (Sf21) cells were maintained and infected by standard procedures (O'Reilly et al (1992); Dracheva, S., et al (1995) *J. Biol. Chem. 270*, 14148-14153) using medium supplemented with 5% heat-inactivated fetal bovine serum

(Hyclone) and 50 μg/ml gentamicin sulfate (Life Technologies, Inc.). All purification procedures were performed at 4°C. Cytosolic extracts of TRAF(NC)-CA21 baculovirus-infected Sf21 cells were prepared as described (Dracheva et al (1995)), without the addition of ATP or $MgCl_2$, frozen under liquid nitrogen, and stored at -80°C. Saturated ammonium sulfate was added to 43% v/v with mixing and incubated at 0°C for 2 hr. Precipitated proteins were harvested by centrifugation, and the pellet was resuspended in buffer A (20 mM HEPES, pH 7.0, 200 mM NaCl, 1 mM DTT, 10% v/v glycerol, 0.1 mM EDTA, 0.1 mM EGTA, and 1 mM PMSF) with 200 mM NaCl. The NaCl concentration was adjusted to 100 mM by dilution with buffer A, and the sample was applied to coupled Source 15S and Source 15Q columns (Amersham Pharmacia Biotech) equilibrated in buffer A with 80 mM NaCl. The material flowing through both columns was collected and applied to a Ceramic Hydroxyapatite (Type II) column (BioRad) equilibrated in buffer B (50 mM potassium phosphate pH 6.2, 100 mM NaCl, 0.2 mM DTT, 1 mM PMSF). Proteins were eluted with a 0 to 55 % gradient of buffer C (500 mM potassium phosphate pH 6.2, 0.2 mM DTT). Peak fractions were pooled and purified proteins were quantitated as described (Gill, S. C., and von Hippel, P. H. (1989) *Anal. Biochem. 182*, 319-326), frozen in aliquots under liquid nitrogen, and stored at -80°C. Expression of CD40c in *Escherichia coli* strain BL21 (DE3) was by induction with 1.0 mM IPTG for 3 h at 37°C. Harvested cell paste was resuspended in 2 volumes of lysis buffer (20 mM HEPES, pH 7.5, 200 mM NaCl, 1 mM DTT, 1 mM EDTA, 1 mM EGTA, 10% v/v glycerol, 1 mM PMSF, 4 μg/ml leupeptin, 4 μg/ml pepstatin A), frozen under liquid nitrogen, and stored at -80°C. Thawed cell paste was resuspended in an equal volume of lysis buffer, and cells were disrupted by nitrogen cavitation. Extracts were clarified by ultracentrifugation for 75 min at 100,000 x g. Saturated ammonium sulfate was added to 66% v/v with mixing and incubated at 0°C for 2 hr. Precipitated proteins were harvested by centrifugation, and the pellet was resuspended in buffer A with 200 mM NaCl. After dialysis overnight in buffer A with 100 mM NaCl, the sample was applied to a Source 15S column (Amersham Pharmacia Biotech) equilibrated in buffer A in 80 mM NaCl. Proteins were eluted with a 5 to 55 % gradient of buffer A with 2 M NaCl Peak fractions were pooled and purified protein was quantitated using a Micro

BCA assay (Pierce Chemical Co.) relative to GST-CD40c (26) as a standard, frozen in aliquots under liquid nitrogen, and stored at -80°C.

Purified CD40c (1.1 mg/ml) was conjugated to biotin in 0.1 M sodium bicarbonate by the addition of D-biotinoyl- -aminocaproic acid-N-hydroxysuccinimide ester (Boehringer Mannheim) at a final concentration of 60 μg/ml. Reagent was removed by dialysis against 40 mM HEPES, pH 7.5, 0.1 M NaCl, 1 mM $MgCl_2$, 0.1 mM DTT. Incorporation of biotin was quantitated by mass spectroscopy and was either one or two mol biotin per mol CD40c. Approximately half of the CD40c remained unconjugated. Biotin-CD40c was titrated in the flashplate assay (below) to empirically determine optimal assay concentrations.

Example 3

CA21 Cell Line

The CA21 cell line producing a mouse IgG1 monoclonal antibody against a peptide epitope was grown and purified as described. Kahn et al (1994); Dracheva et al (1995). Specifically, production of CA21 monoclonal antibody is performed as follows: CA21 hybridoma cell lines producing monoclonal antibodies directed against the cytoplasmic domain of L-selectin were prepared by hyperimmunization of BALB/c mice with a synthetic peptide corresponding to the entire cytoplasmic domain of L-selectin, as described in Kishimoto, T. K., (1990) *Proc. Natl. Acad. Sci. USA 87*, 2244-2248, incorporated herein by reference. Spleen cells were fused with the Sp2/O-Ag14 myeloma fusion partner. Reference in this regard can be made to Shulman, M., et al. (1978) *Nature 276*, 269–270; Mandal, C. et al. (1991) *Hybridoma 10*, 459–466; and Norris, S. H., et al. (1991) *J. Pharm. Biomed. Anal. 9*, 211–217, the entire contents of each are herein incorporated by reference. Hybridoma supernatants were screened for the ability to specifically recognize immobilized cytoplasmic domain peptide. Positive clones were further screened for the ability to immunoprecipitate L-selectin. CA21 (IgG1) monoclonal antibody was purified by protein G affinity chromatography.

Example 4

Fab Fragments

Fab fragments were prepared by protease digestion by standard methods (Peters. J. H.. and Baumgarten. H. (1992) *Monoclonal antibodies*. Springer-Verlag, p. 276) and iodinated by the IODO-GEN® method. Millar, W. T.. and Smith J. F. B. (1983) *Int. J. Appl. Radiat. Isot. 34*. 639-641. Radiolabeled Fab fragment was purified by gel filtration on Sephadex G50-50 and stored at 4°C in 1% BSA (Sigma) in Dulbecco's phosphate buffered saline (D-PBS), pH 7.4 (GIBCO-BRL).

Example 5

Flashplate Assay

Biotin-CD40c (0.5 μg/ml) was bound to streptavidin-coated 96-well scintillant-embedded plates (Flashplates, New England Nuclear. catalog #15112) overnight at 4°C in D-PBS, 0.1 mM DTT, 0.01% BSA (100 μl/well). Plates were blocked for 2 hr at room temperature by the addition of Dulbecco's-PBS, 0.1 mM DTT, 1.0% BSA (100 μl/well). In a separate 96-well round bottom polypropylene plate peptide inhibitors were diluted in 40 mM HEPES, pH 7.5, 0.1 M NaCl, 1.0 mM $MgCl_2$, 0.1 mM DTT, 0.01% BSA, and TRAF2(NC)-CA21 was added at a final concentration of 5 μg/ml. Plates were preincubated for 30 min at room temperature. After washing the biotin-CD40c-coated flashplate three times with D-PBS, 0.1 mM DTT, inhibitor-TRAF2(NC)-CA21 mixtures were transferred from the preincubation plate to the washed plate (100 μl/well). Alternatively, after washing the biotin-CD40c-coated flashplates, inhibitors were added directly without TRAF preincubation in a final volume of 50 μl 40 mM HEPES, pH 7.5, 0.1 M NaCl, 1.0 mM $MgCl_2$, 0.1 mM DTT, 0.01% BSA, and 50 μl/well TRAF2(NC)-CA21 (10 μg/ml stock to make a final concentration of 5 μg/ml) was added. Flashplates were incubated for 1 hr at room temperature, washed three times with 40 mM HEPES, pH 7.5, 0.1 M NaCl, 1.0 mM $MgCl_2$, 0.1 mM DTT, and 100 μl/well of [^{125}I]CA21 Fab fragment at an adjusted specific activity of 0.08 μCi/well added in 40 mM HEPES, pH 7.5, 0.1 M NaCl, 1.0 mM $MgCl_2$, 0.1 mM DTT, 0.1% BSA. After a 1 hr incubation at room temperature plates were sealed and counted in a TopCount scintillation counter (Packard: one minute/well, 2 minute plate delay).

Example 6

Peptide Inhibitors

Peptides (5 mg each) were purchased from AnaSpec (San Jose, CA) and weresynthesized N-terminally acetylated and C-terminally amidated. Peptides were purified to >95% purity as judged by quantitative HPLC analysis and mass spectroscopic analysis.

WE CLAIM:

1. A quantitative assay for measuring the ability of a substance to effect binding of a TRAF protein to a TRAF interacting receptor, the assay comprising:

providing the receptor having a cytosolic binding domain or fragment thereof, wherein the receptor is bound to a multi-well plate, the multi-well plate being capable of allowing formation of a multimeric receptor and being capable of allowing detection of a signal; providing a TRAF protein or fragment thereof possessing a terminal tag, the tag comprising one or more amino acids and being capable of binding a signal-generating antibody or fragment thereof; providing the substance; combining the receptor bound to the well, the TRAF protein and the substance; and detecting the signal-generating antibody.

2. The assay according to claim 1 wherein the TRAF protein or fragment thereof is selected from the group consisting of TRAF1, TRAF2, TRAF3, TRAF4, TRAF5 and TRAF 6.

3. The assay according to claim 2 wherein the TRAF interacting receptor is selected from the group consisting of ATAR, LT-ßR, TNFR2, CD40, CD30, OX-40 and 4-1BB.

4. The assay according to claim 2 wherein the TRAF interacting receptor is conjugated with biotin and the multi-plate well is coated with streptavidin.

5. The assay according to claims 3 or 4 wherein the multi-well plate comprises a scintillant and the signal-generating antibody possesses a radioactive isotope.

6. The assay according to claim 5 wherein the TRAF protein or fragment thereof is TRAF2 or TRAF3 and the TRAF interacting receptor is CD40.

7. The assay according to claim 1 wherein the TRAF protein fragment consists substantially of Sequence ID# 1 or Sequence ID# 2.

8. The assay according to claim 1 wherein the substance is added directly to the well.

9. The assay according to claim 1 wherein the substance is preincubated with the TRAF protein.

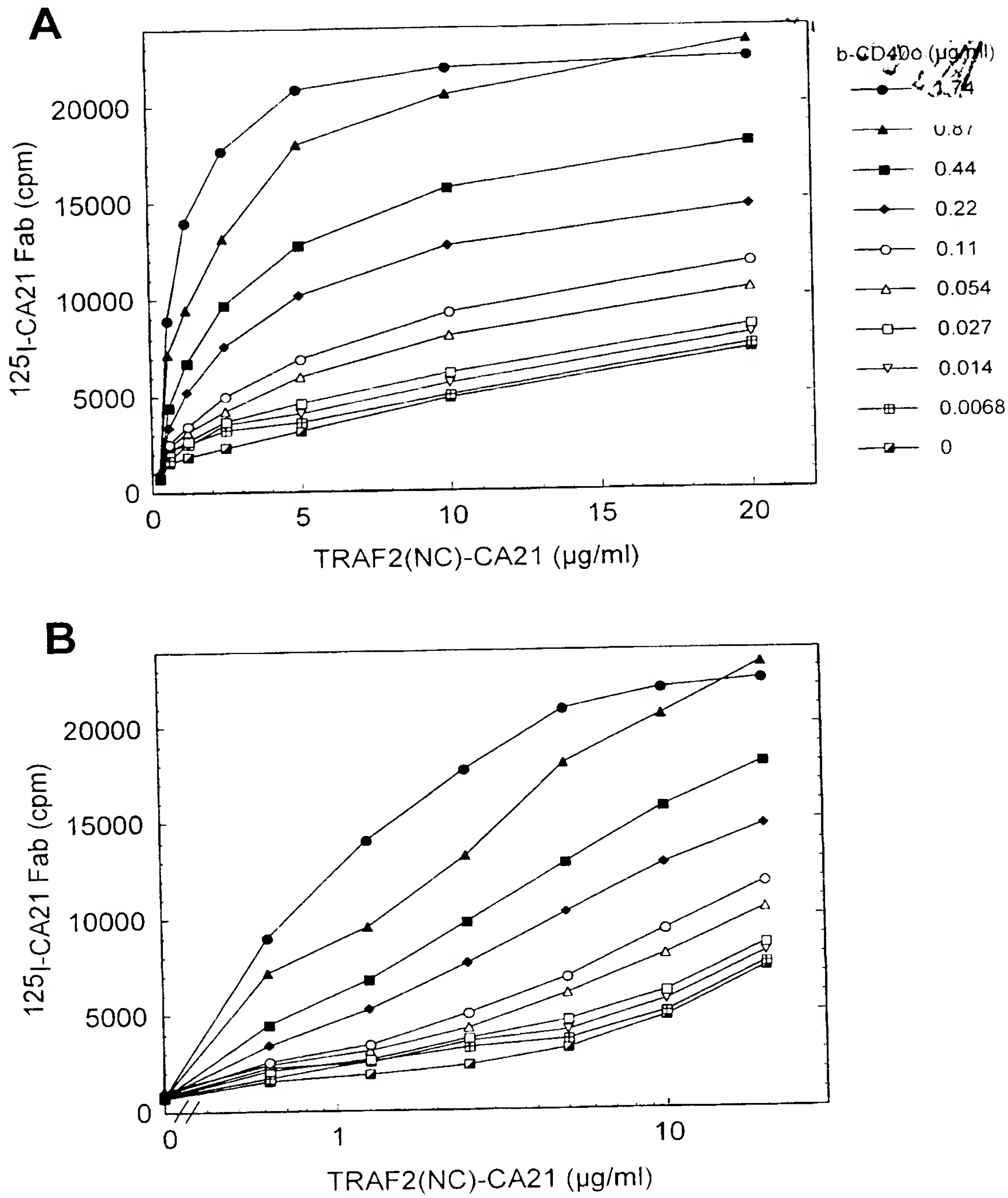
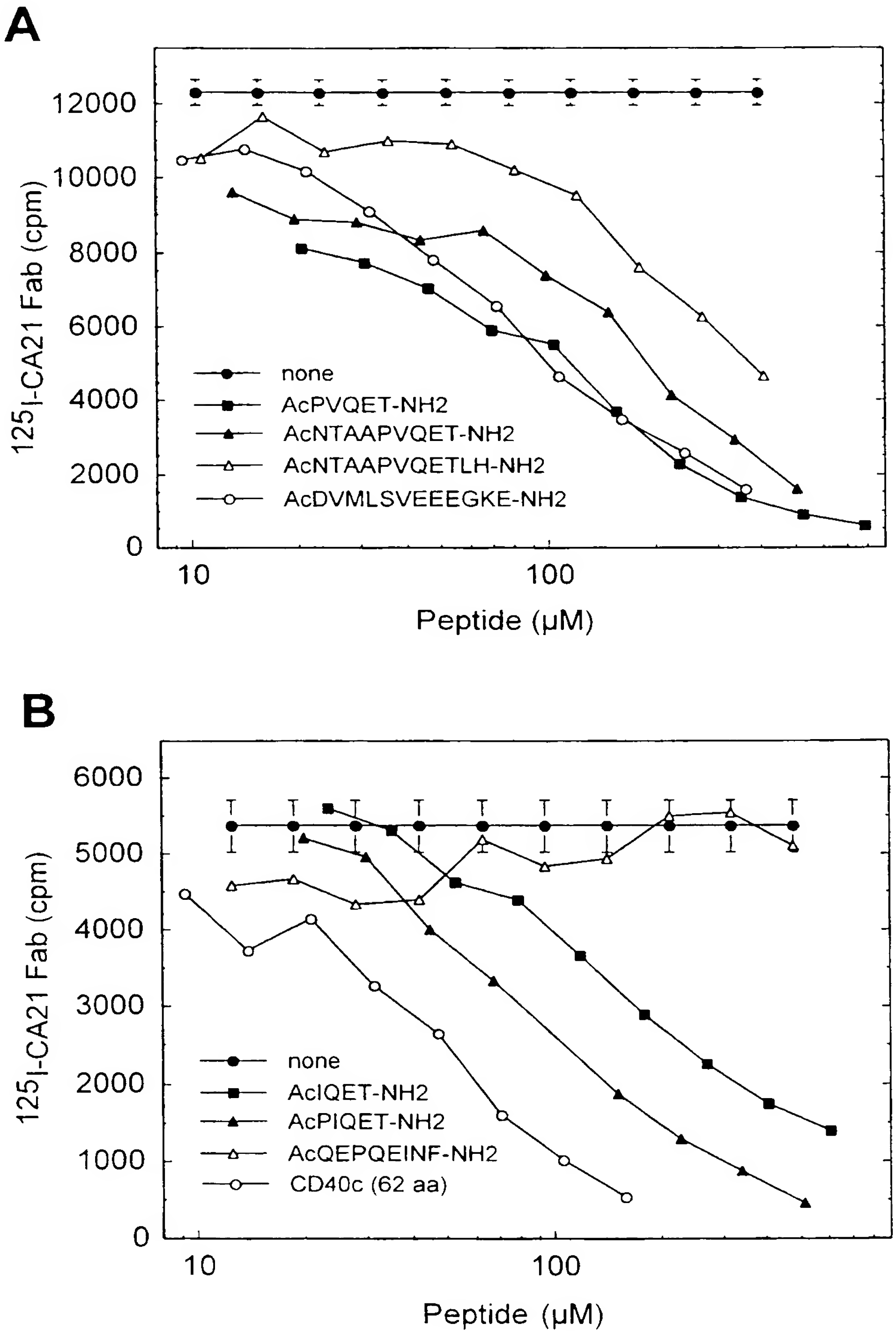

A
125I-CA21 Fab (cpm)
0
5000
10000
15000
20000
0
5
10
15
20
TRAF2(NC)-CA21 (μg/ml)
b-CD40o (μg/ml)
1.74
0.87
0.44
0.22
0.11
0.054
0.027
0.014
0.0068
0
B
125I-CA21 Fab (cpm)
0
5000
10000
15000
20000
0
1
10
TRAF2(NC)-CA21 (μg/ml)

Figure 1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8000
6000
4000
2000
0
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AcNTAAPVQET-NH2
AcNTAAPVQETLH-NH2
AcDVMLSVEEEGKE-NH2
10
100
Peptide (μM)
B
6000
5000
4000
3000
2000
1000
0
125I-CA21 Fab (cpm)
none
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AcPIQET-NH2
AcQEPQEINF-NH2
CD40c (62 aa)
10
100
Peptide (μM)

Figure 2

SEQUENCE LISTING

<110> Kehry, Marilyn R
Pullen, Steven S
Crute, James J

<120> A High Throughput Compatible Assay for Receptor-TRAF Interactions

<130> 9/142 PCT Nucl.Seq

<140>
<141>

<160> 2

<170> PatentIn Ver. 2.0

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<211> 243
<212> PRT
<213> human

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35 40 45

Lys Val Gln Gln Leu Glu Arg Ser Ile Gly Leu Lys Asp Leu Ala Met

50 55 60

Ala Asp Leu Glu Gln Lys Val Leu Glu Met Glu Ala Ser Thr Tyr Asp

65 70 75 80

Gly Val Phe Ile Trp Lys Ile Ser Asp Phe Ala Arg Lys Arg Gln Glu

85 90 95

Ala Val Ala Gly Arg Ile Pro Ala Ile Phe Ser Pro Ala Phe Tyr Thr

100 105 110

Ser Arg Tyr Gly Tyr Lys Met Cys Leu Arg Ile Tyr Leu Asn Gly Asp

115 120 125

Gly Thr Gly Arg Gly Thr His Leu Ser Leu Phe Phe Val Val Met Lys

130 135 140

Gly Pro Asn Asp Ala Leu Leu Arg Trp Pro Phe Asn Gln Lys Val Thr

145 150 155 160

Leu Met Leu Leu Asp Gln Asn Asn Arg Glu His Val Ile Asp Ala Phe

165 170 175

Arg Pro Asp Val Thr Ser Ser Ser Phe Gln Arg Pro Val Asn Asp Met
180 185 190

Asn Ile Ala Ser Gly Cys Pro Leu Phe Cys Pro Val Ser Lys Met Glu
195 200 205

Ala Lys Asn Ser Tyr Val Arg Asp Asp Ala Ile Phe Ile Lys Ala Ile
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Asp Pro Tyr

<210> 2

<211> 228

<212> PRT

<213> human

<400> 2

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1 5 10 15

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20 25 30

Ser Arg His Asp Gln Met Leu Ser Val His Asp Ile Arg Leu Ala Asp

35 40 45

Met Asp Leu Arg Ile Gln Val Leu Glu Thr Ala Ser Tyr Asn Gly Val

50 55 60

Leu Ile Trp Lys Ile Arg Asp Tyr Lys Arg Arg Lys Gln Glu Ala Val

65 70 75 80

Met Gly Lys Thr Leu Ser Leu Tyr Ser Gln Pro Phe Tyr Thr Gly Tyr

85 90 95

Phe Gly Tyr Lys Met Cys Ala Arg Val Tyr Leu Asn Gly Asp Gly Met

100 105 110

Gly Lys Gly Thr His Leu Ser Leu Phe Phe Val Ile Met Arg Gly Glu

115 120 125

Tyr Asp Ala Leu Leu Pro Trp Pro Phe Lys Gln Lys Val Thr Leu Met

130 135 140

Leu Met Asp Gln Gly Ser Ser Arg Arg His Leu Gly Asp Ala Phe Lys

145 150 155 160

Pro Asp Pro Asn Ser Ser Ser Phe Lys Lys Pro Thr Gly Glu Met Asn

165 170 175

Ile Ala Ser Gly Cys Pro Val Phe Val Ala Gln Thr Val Leu Glu Asn

180 185 190

Gly Thr Tyr Ile Lys Asp Asp Thr Ile Phe Ile Lys Val Ile Val Asp
195 200 205

Thr Ser Asp Leu Pro Asp Pro Ala Ser Ala Ser Ser Lys Arg Ser Met
210 215 220

Asn Asp Pro Tyr
225

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/19272

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N33/566 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PULLEN SS, MILLER HG, EVERDEEN DS, DANG TTA, CRUTE JJ, KEHRY MR: "CD40-tumor necrosis factor receptor-associated factor (TRAF) interactions: regulation of CD40 signaling through multiple TRAF binding sites and TRAF hetero-oligomerization" BIOCHEMISTRY, vol. 37, no. 34, 25 August 1998 (1998-08-25), pages 11836-18845, XP002129046 cited in the application page 11838 ---	1-9
P,Y	EP 0 915 155 A (MOCHIDA PHARM CO LTD) 12 May 1999 (1999-05-12) paragraph '0058! - paragraph '0063!	1-9
Y	& WO 97 38099 A (MOCHIDA PHARM) ---	

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 January 2000	11/02/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Hart-Davis, J

1

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
Y	US 5 767 244 A (GOEDDEL DAVID V ET AL) 16 June 1998 (1998-06-16) claim 4 ---	1-9
A	ISHIDA T, MIZUSHIMA SI, AZUMA S, KOBAYASHI N, TOJO T, SUZUKI K, AIZAWA S, WATANABE T, MOSIALOS G, KIEFF E, YAMAMOTO T, INOUE J: "Identification of TRAF6, a novel tumor necrosis factor receptor-associated factor protein that mediates signaling from an amino-terminal domain of the CD40 cytoplasmic region" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 271, no. 46, 15 November 1996 (1996-11-15), pages 28745-28748, XP002129047 cited in the application the whole document ---	1
A	ISHIDA TK, TOJO T, AOKI T, KOBAYASHI N, OHISHI T, WATANABE T, YAMAMOTO T, INOUE J: "TRAF5, a novel tumor necrosis factor receptor-associated factor family protein, mediates CD40 signaling" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 93, September 1996 (1996-09), pages 9437-9442, XP002129048 cited in the application the whole document ---	1
A	SATO T, IRIE S, REED JC: "A novel member of the TRAF family of putative signal transducing proteins binds to the cytosolic domain of CD40" FEBS LETTERS, vol. 358, no. 2, 23 January 1995 (1995-01-23), pages 113-118, XP002129049 cited in the application the whole document ---	1
A	GEDRICH RW, GILFILLAN MC, DUCKETT CS, VAN DONGEN JL, THOMPSON CB: "CD30 contains two binding sites with different specificities for members of the tumor necrosis factor receptor-associated factor family of signal transducing proteins" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 271, no. 22, 31 May 1996 (1996-05-31), pages 12852-12858, XP002129050 cited in the application the whole document ---	1

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/19272

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
A	HU HM, O'ROURKE K, BOGUSKI MS, DIXIT VM: "A novel RING finger protein interacts with the cytoplasmic domain of CD40" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 269, no. 48, 2 December 1994 (1994-12-02), pages 30069-30072, XP002129051 cited in the application the whole document -----	1

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 99/19272

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0915155 A	12-05-1999	AU 2307697 A	29-10-1997
		CA 2251578 A	16-10-1997
		WO 9738099 A	16-10-1997
US 5767244 A	16-06-1998	US 5710013 A	20-01-1998
		AU 706887 B	01-07-1999
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		CA 2250425 A	30-10-1997
		EP 0895545 A	10-02-1999
		WO 9740192 A	30-10-1997